Journal of Medicinal and Nanomaterials Chemistry 4 (2022) 313-326

SPC

Journal of Medicinal and Nanomaterials Chemistry

Journal homepage: https://jmnc.samipubco.com/

Original Research Article

Promising anti-inflammatory bio-efficacy of saponin loaded silver nanoparticles prepared from the plant *Madhuca longifolia*

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ARTICLE INFORMATION

Received: 02 September 2022
Received in revised: 11 October 2022
Accepted: 16 October 2022
Available online: 01 December 2022

DOI: 10.48309/JMNC.2022.4.5

KEYWORDS

Madhuca Longifolia
Green Synthesis
Saponin Loaded Silver Nanoparticles
Enhanced Anti-Inflammatory Activity

ABSTRACT

Phyto-compounds facilitated synthesis of nanoparticles has created an exceptional impact in the formation of nanoparticles and is used for the synthesis of modern nano drugs. Ignorance about phytochemical composition particularly knowledge of the bio-active principle of medicinal plant restricts the demonstration of the real picture of the enhancement of any bio-efficacy. The present communication scientifically established anti-inflammatory bio-efficacy in seeds of the folk plant *Madhuca longifolia* and its significant enhancement by bio-active principle (saponin) loaded silver nanoparticles (S@AgNp$_s$). A family of four saponins has been explored quantified (3.59%) and characterized (Micro Mass ESI-TOF MS spectra). Synthesis of S@AgNp$_s$ has been conducted in a green single step and thoroughly characterized. *In- vivo* assessment of antiinflammatory bio-efficacy has been carried out using carrageenan induced hind paw edema in *Swiss albino* mice model. Anti-inflammation bio-efficacy of native seed extract (15 mg/kg/bw) was found 46.84% which was further elevated and further rose to 56.10% by saponin at considerable low optimized dose (1.5 mg/kg/bw). Anti-inflammatory bio-efficacy was further successfully enhanced to (70.99%) by S@AgNp$_s$, almost close to that of reference drug (Diclofenac sodium; 76.42%). Saponin loaded silver nanoparticles (S@AgNp$_s$) prepared from the seed extract of the plant *M. longifolia* seem to be an ideal candidate for the development of complimentary herbal nanomedicine for antiinflammation.

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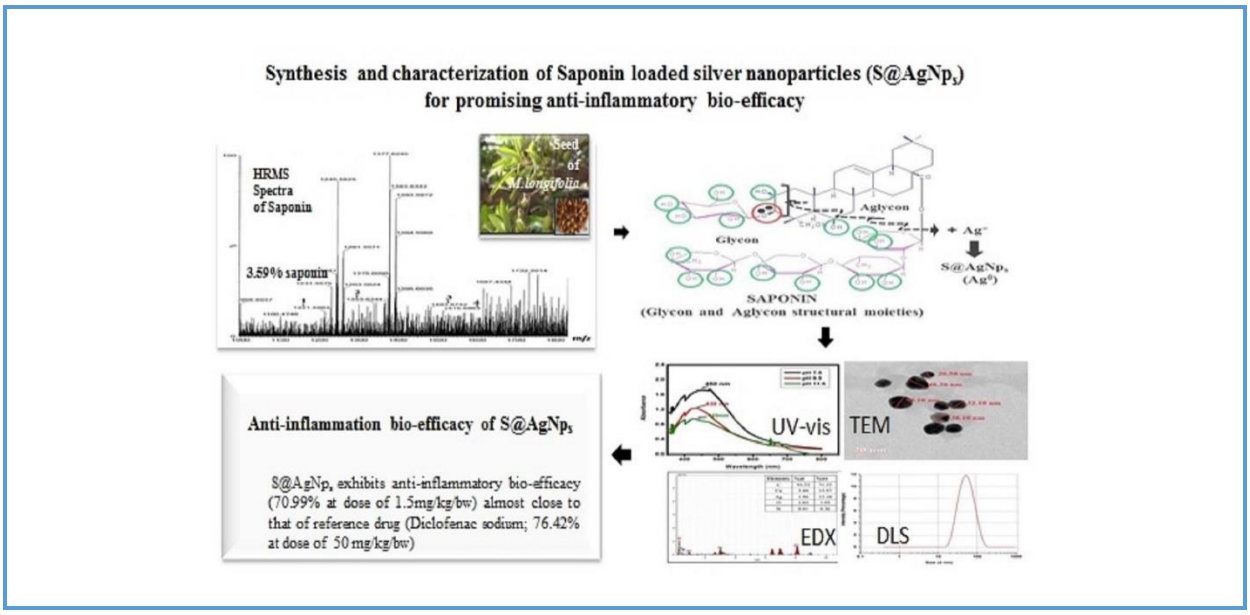
Graphical Abstract



Introduction

Several tactics are available for the synthesis of metal nanoparticles *viz* electrochemical, microwave, sonochemical, phase transfer and photochemical synthesis [1-3]. These methods are over-priced and also require chemicals which ultimately lead to health risks. Chemically synthesized metal nanoparticles show an enormous range of side effects like hepatic problems and metabolic disorders which are quite annoying [4]. Numerous variety of metal nanomaterials are being acquired using copper, zinc, titanium, magnesium, gold, and silver [5-7]. Green approaches for the synthesis of metal nanoparticles using plants and microorganisms are becoming highly popular [8, 9]. However, multiple purification steps and sophisticated processes are required in the microbialbased synthesis of nanoparticles [10]. Phyto-compounds facilitated synthesis of nanoparticles has received an unprecedented significance and becomes one of the thrust areas of medicinal research [11-13]. Ample reports are available on the use of medicinal plants in the synthesis of silver and gold nanoparticles [14, 15]. Among the noble metals, silver has been a focus of interest particularly for anti-inflammatory drugs [16]. Silver itself has potent antimicrobial activity including antifungal, anti-oxidant and anti-inflammatory [17]. Lack of knowledge on the phytochemical constituents of the plant extract, ruin the fate of entire prospects of the study. Thus characterization of a proper phytochemical as the bio-active principle is essentially required. In the current scenario, bio-active principle loaded metal nanoparticles having enhanced bio-efficacy have been found highly attractive [18,19].

Inflammation, although appears as a simple ailment but now seeking serious attention all over the world. It is a perplexing process which is affiliated with alternation in vascular permeability, physiological change, denaturation of proteins, an imbalance in enzymes and hormones [20]. Several conventional anti-inflammatory drugs are common in use but are assorted with side effects like kidney, heart and liver failure [21].

Little efforts have been made towards synthesis and characterization of metal nanoparticles, loaded with the bioactive principle of medicinal plants having anti-inflammatory properties.

Madhuca longifolia (Mahua) is an indigenous plant belongs to the Sapotaceae family and has been reported to have several nutritional potentials [22]. It is generally valued for its seed which has an abundant amount of oil-bearing capacity for which about 0.14 million ton of seeds are produced in India [23]. Apart from its nutritional values, seeds have not been much investigated for medicinal uses and remained unseen in the eyes of the researchers. In the tribal's life, the plant *M. longifolia* is frequently used as folk medicine for wound healing, inflammation or swelling [24, 25].

The present communication for the first time reports the promising antiinflammatory bio-efficacy of S@AgNp$_s$, prepared from the extracted saponin of the seeds of the plant *M.longifolia*. *In- vivo* experiments have been carried out in carrageenan-induced hind paw edema in *Swiss albino* mice model. Isolation of saponin (bio-active principle) from aq. alc. seed extract has been carried out and characterized using high-resolution mass spectrum (Micro Mass ESI-TOF MS spectrometer). S@AgNp$_s$ exhibited excellent anti-inflammatory bio-efficacy almost close to that of reference drug (Diclofenac sodium).

Experimental

Collection and identification of seeds

Seeds of *Madhuca longifolia* were collected from the local area of Rajaborari, Madhya Pradesh, India and were identified by Taxonomy Division of Department of Botany, Dayalbagh Educational Institute Agra, India.

Microwave-Ultrasound assisted extraction of seeds

Defatted seed powder (100 g) was treated in aqueous ethanol (300 mL) in a beaker and placed in a microwave oven (3 min) with a maximum output power of 1150 W and frequency of 2.45 GHz and cooled. The sample was transferred to an ultrasonic bath and sonicated for 40 min at room temperature. The suspension was cooled and filtered.

Isolation, characterization and quantitative estimation of saponin

Seed extract (20 mL) was treated with twofold fractions of diethyl ether (40 mL) and finally transferred into a separating funnel. The ethereal layer was discarded and the aqueous layer was extracted twice with a biphasic solvent mixture (n-butanol: 5% NaCl; 6:1) in a separating funnel. The nbutanol layer was heated in a water bath for 30 min, dried in a crucible and finally weighed. A mixture of saponins was characterized using high-resolution spectrometer (Micro Mass ESI-TOF MS). Recording of spectra involved ionization of sample (2 µl) with ethanol through a small heated capillary (flow rate of 1-10 µl min^{-1}) in negative mode [M-H$^-$].

Synthesis of saponin loaded silver nanoparticles (S@AgNps)

Synthesis of silver nanoparticles was carried out at different pH, varying concentration of silver nitrate solution and saponin. Optimized experimental conditions for the synthesis of S@AgNp$_s$ were as follows: saponins (1 mL; 1.5 mg /mL), silver nitrate solution (10 mL; 1 mM) and sonication (45 min; 20 KHz) at pH 11.5. The formation of S@AgNp$_s$ was observed by the change in color from pale yellow to dark brown.

Characterization of S@AgNps

S@AgNp$_s$ were characterized using Ultra Violet–visible spectroscopy (UV-vis3000$^+$ Lab

India), X-ray diffraction (Bruker AXS D8 Advance, Germany), Field emission scanning electron microscopy (Nova Nano FE-SEM 450, Netherland), Transmission electron microscopy (at magnification of 300,000X), Energy dispersive X-ray spectroscopy (Tecnai G2 T 20 ST, Germany) Dynamic light scattering (Nano ZS90 model Malvern, Germany) and Fourier transform Infrared spectroscopy (Agilent Cary 630 FTIR, India).

Anti-inflammatory in-vivo bioassay

Male *Swiss albino* mice (weight: 25-30 g) were obtained from animal house of Jawaharlal Nehru Cancer & Research Centre Bhopal, Madhya Pradesh and used for the evaluation of *in-vivo* acute toxicity (Ethical permission; CPCSEA Registration No. 500/01/9/CPCSEA/2001). Animals were kept at a temperature of 25-28 °C in clean polypropylene cages with 12 h light and dark cycle with proper pellet diet and water *ad libitum*. Mice were monitored at the one-time exposure of dose ranging (1000-4500 mg/kg/bw) for the evaluation of LD_{50} of $S@AgNp_s$.

In-vivo anti-inflammatory bioassay in *Swiss albino* mice has been carried out. Carrageenan acute hind paw edema was produced by injecting carrageenan (1% suspension in sterile normal saline; 0.1 mL) locally into the planter aponeurosis of the right hind paw of mice [26]. Mice were divided into ten groups, each consisting of six animals. Group I treated as a control group. Group II was given reference drug Diclofenac sodium (50 mg/kg/bw). Groups III, IV, V and VI were given seed extract at the doses of (5, 10, 15 and 20 mg/kg/bw).

Groups VII, VIII, IX were given saponin (1.0, 1.5, 3.0 mg/kg/bw). Group X was given $S@AgNp_s$ (1.5 mg/kg/bw). After half an hour, the carrageenan (0.1 mL; 1%) was injected into the right hind paw of each mouse. The volume of the paw edema was measured by plythesmometre (at 0.5, 1, 2 and 4 h). Percent inhibition in each case was calculated using the formula:

$$\% \, Inhibition = (Vc - Vt) \div Vc \times 100,$$

where, Vc = mean increase in paw edema volume in control group; Vt = mean increase in paw edema volume in the treated group.

Statistical analysis

Results were expressed as the mean ± standard deviation. Data were analyzed by one way ANOVA (Tukey-Kramer) using Graph pad Insat software. Significance of the values was considered at $P<0.001$.

Results and Discussion

It is evidenced that occurrence of the saponin as plant secondary metabolites are mainly responsible for anti-inflammatory property [27, 28]. Detailed phytochemical studies of the plant *M.longifolia* is lacking. The fact has motivated us to isolate and characterize saponin in the seed extract of *M.longifolia* plant. The comparison of antiinflammatory bio-efficacy of native seed extract, saponin and saponin loaded silver nanoparticles ($S@AgNp_s$) has not only ascertained the important role of saponin as a bio-active principle but also highlighted the extent of green nanotechnological enhancement in the anti-inflammatory bioefficacy.

Micro Mass ESI-TOF MS chromatogram exhibited the presence of a family of four saponins (3.59%). Micro Mass ESI-TOF MS chromatogram (Figure 1) ascertained the presence of *Mi-saponin A (1)*; m/z 1221.5963, *Mi-saponin B (2)*; m/z 1353.6344, *Madhucoside A (3);* m/z 1483.6742 and *Madhucoside B (4);* m/z 1515.6863 along with some unassigned peaks of non- saponin contents.

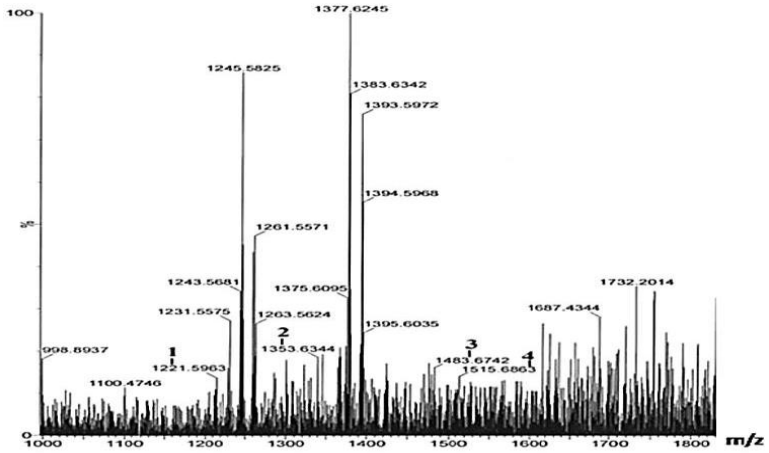


Figure 1. Micro Mass ESI-TOF MS chromatogram of saponin extract

At pH, 11.5 saponin started reducing silver nitrate to nano silver (S@AgNp$_s$). Saponin consists of structurally-related steroid or triterpenoid aglycone (sapogenin) linked to sugar moieties through glycosidic linkage. The oxygen atom of a glycosidic linkage having a lone pair of electrons is likely to reduce Ag^+ ions into Ag^0, forming S@AgNp$_s$. Further, hydroxyl groups of the sugar moieties and sapogenin might also play a favorable role in the reduction of Ag^+ ions [29]. The oxidized saponin being electron deficient in nature may impart free radical scavenging abilities, acting as an anti-oxidant. Thus saponin, not only forms silver nanoparticles but also get involved in its simultaneous loading or capping on silver nanoparticles. This medicated coating on the freshly generated silver nanoparticles and enhance pharmacological efficacy provides robust shielding from aggregations, keeping them in nano state (stability). Hence, the overall strong synergistic reduction potential of saponin (Figure 2) is capable of forming silver nanoparticles, acting as reducing and capping agents.

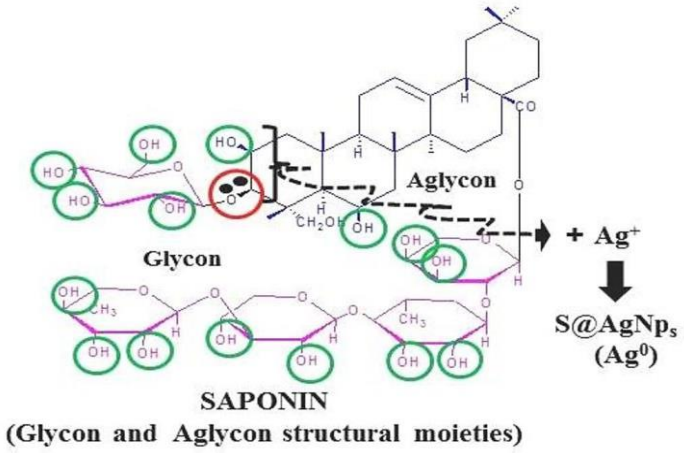


Figure 2. The proposed chemical reaction of saponin (glycosidic oxygen and –OH groups) with Ag+ ions rendering the formation of S@AgNps

Thorough characterizations of synthesized S@AgNp$_s$ were carried out as follows:

Ultra Violet–visible spectroscopy

Synthesis of S@AgNp$_s$ was carried out at different concentrations (10^{-4}-10^{-2} M) of silver nitrate. At concentration 10^{-4} M of silver nitrate, no perceptible surface plasmon resonance (SPR) peak appeared in the desired range of 400–450 nm. At silver nitrate concentrations (10^{-3} M and 10^{-2} M), SPR peaks were found at 420 nm and 440 nm respectively. The absorbance of SPR peak at silver nitrate higher concentration (10^{-3} M) was of much higher intensity and, therefore, considered optimum concentration for the synthesis of S@AgNp$_s$.

S@AgNp$_s$ were synthesized using saponin and silver nitrate solution in different proportion as functions of pH 7.5, 9.5 and 11.5 (Figure 3). S@AgNp$_s$ fabricated at pH 11.5 was considered optimum, having a characteristic peak at λ_{max}: 420 nm. No peak was observed at highly acidic conditions. Interestingly, optimized nanoparticles exhibited splendid stability over a few weeks.

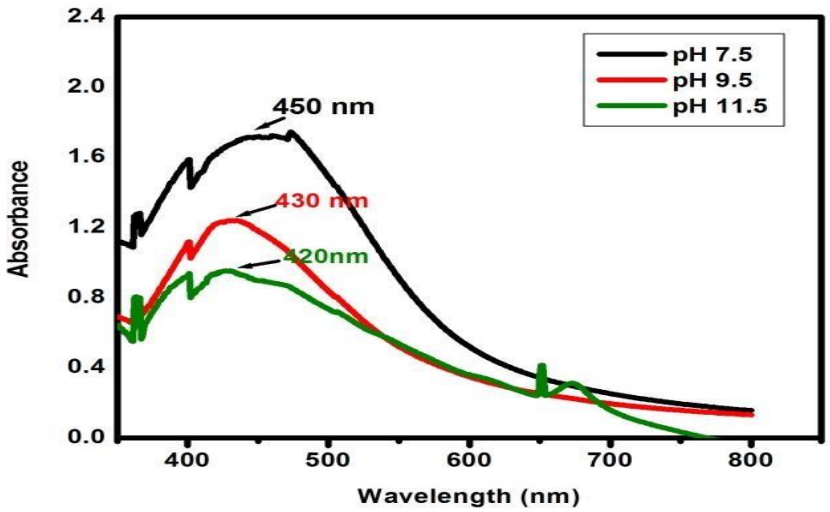


Figure 3. UV–vis spectra of saponin loaded silver nanoparticles at different pH

X-ray diffraction

Bragg reflection peaks for S@AgNp$_s$ appeared at the position of 2θ at 38.11° and 44.27°. The fact indexed to (111) and (200) lattice planes of the face-centered cubic structure of silver nanoparticles (JCPDS file 04-0783). The intensity of the diffraction peak corresponding to (200) crystallographic planes was found to lower than (111). The fact established lattice plane (111) as a transcendent crystallographic plane (Figure 4). The (111) plane is known to be more reactive because of its high atom density [30]. Some unassigned peaks were observed which were due to the crystallization of bioorganic phase [31].

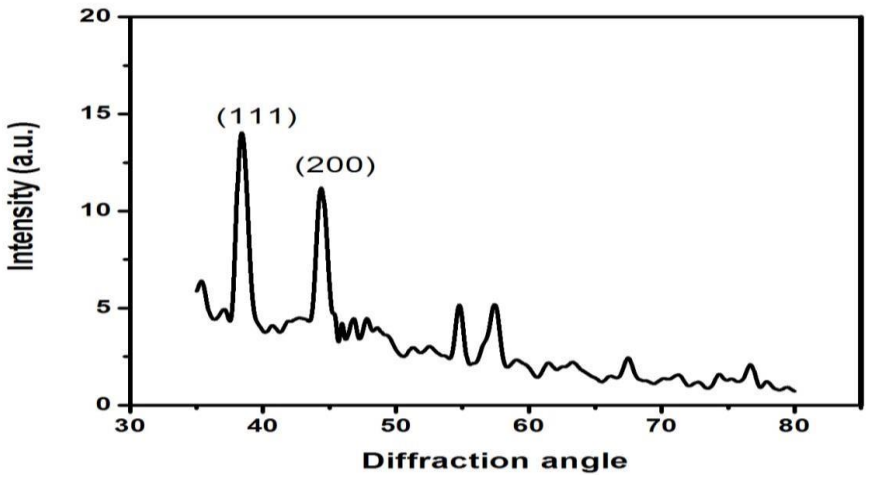


Figure 4. XRD of saponin loaded silver nanoparticles

FE-SEM, TEM, and EDX studies

FE-SEM image (Figure 5) acquired from drop coated films of nanoparticles indicated polydispersed spherical shaped surface morphology of S@AgNp$_s$. TEM analysis confirmed the formation of S@AgNp$_s$ in the size range 20-48 nm. (Figure 6). The morphology of S@AgNp$_s$ was almost spherical in shape with a light-colored coating. The desirable signals of silver metal were found at 3KeV in EDX spectra of the saponin loaded silver nanoparticles (Figure 7). The presence of other peaks may be ascribed to Cu and C which is an artifact of the Cu-grid on which the sample was coated. The peaks of O and N might have initiated from the biomolecules that are adhered to the surface nanoparticles.

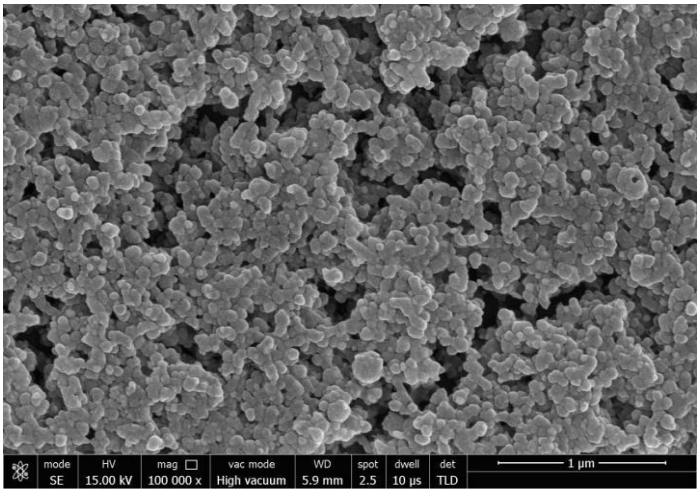


Figure 5. FE-SEM image of saponin loaded silver nanoparticles

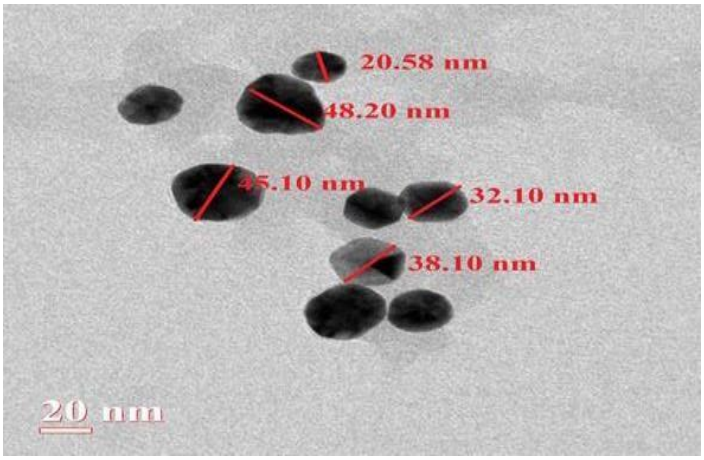
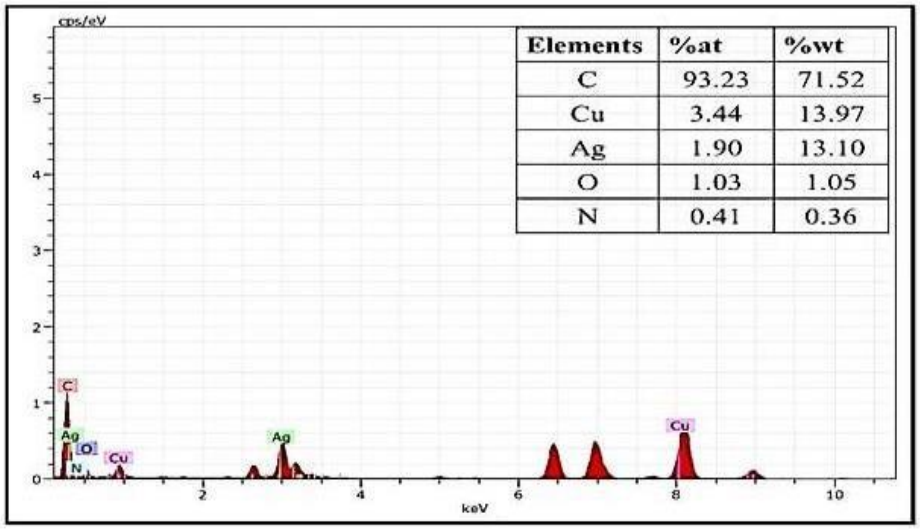


Figure 6. TEM image of saponin loaded silver nanoparticles



Elements	%at	%wt
C	93.23	71.52
Cu	3.44	13.97
Ag	1.90	13.10
O	1.03	1.05
N	0.41	0.36

Figure 7. EDX record of saponin loaded silver nanoparticles

Dynamic light Scattering

Average hydrodynamic size of S@AgNp$_s$ was 52.40 nm in an asymmetric distribution between of 13 to 200 nm (Figure 8). The high intensity distribution of particle size highlighted the fact that fabricated nanoparticles are in quite lower range. Zeta potential of the fabricated S@AgNp$_s$, determined in water as a dispersant, was -33.5 mV (Figure 9). The high negative charge constitutes a repulsive barrier that physically separates the nanoparticles, avoiding aggregation [32]. The fact finds support from TEM images of nanoparticles exhibiting light-colored coating which might be due to the presence of saponin around the nanoparticles, acting as a protective barrier against aggregation [33]

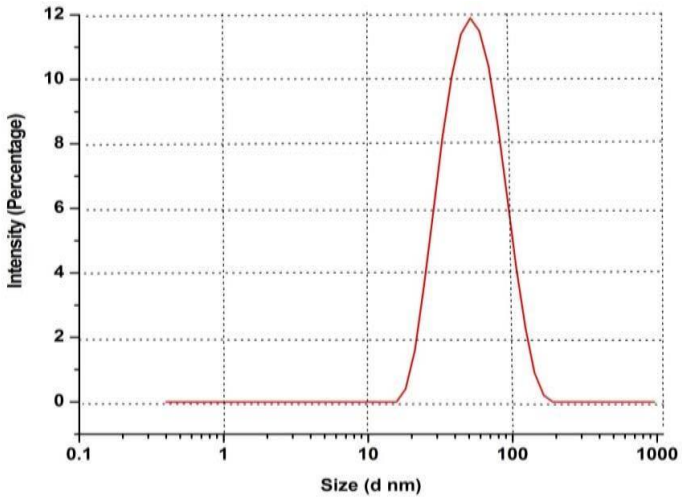


Figure 8. Size distribution of saponin loaded silver nanoparticles

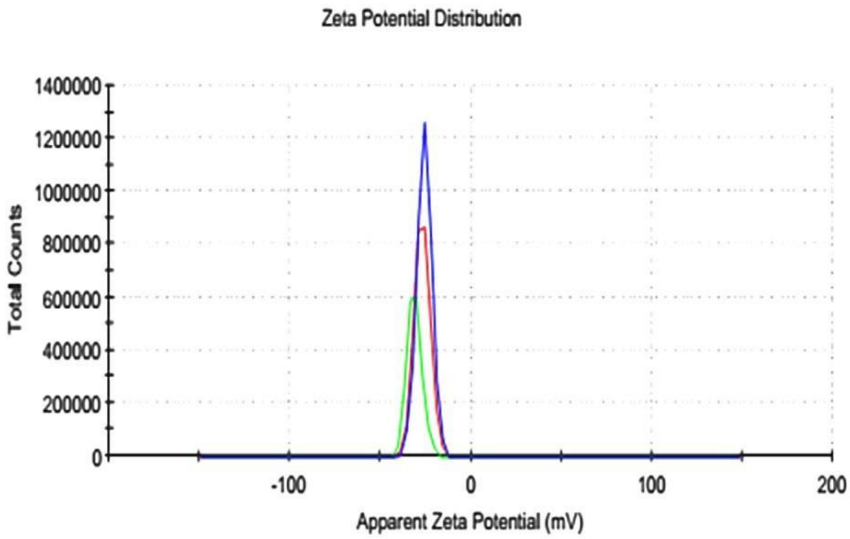


Figure 9. Zeta potential of saponin loaded silver nanoparticles

FTIR analysis

FTIR spectra of native saponin and saponin loaded silver nanoparticles were recorded (Figure 10). Almost overlapping of the peaks, depicting the structure of saponin (OH, -C-H-, -C=C- and -C-O-C-) indicates the presence of residue saponin on the surface of the S@AgNp$_s$. Consequently, the occurrence of these peaks in the FTIR spectra of the S@AgNp$_s$ indicates the dual role of saponin as reducing and stabilizing agents.

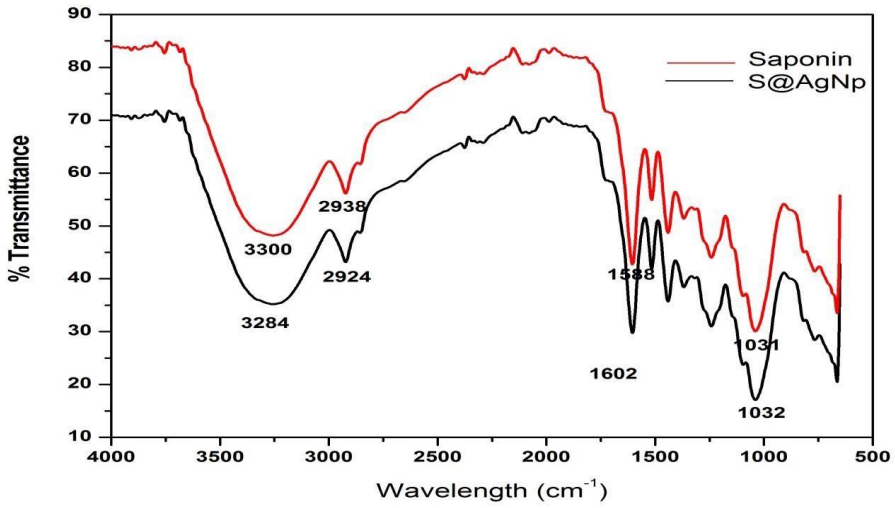


Figure 10. FTIR spectra of saponin and saponin loaded silver nanoparticles

Anti-inflammatory in -vivo bioassay: Carrageenan-induced hind paw edema

Toxicity of S@AgNps was monitored after one-time exposure of the dose ranging (1000-45000 mg/kg/bw). LD50 (Table 1) was found (3000mg/kg bw).

Table1. Mortality percentage of S@AgNp$_s$ in treatment groups

S.No.	Dose (mg/kg/bw)	Time Interval				Mortality (%)
		0-6 h	6-24 h	24-48 h	48-72 h	
1.	1000	-	-	-	-	Nil
2.	1500	-	-	-	-	Nil
3.	2000	-	-	-	-	Nil
4.	2500	-	-	-	-	Nil
5.	3000	-	2	1	-	50.00
6.	3500	-	2	2	-	66.60
7.	4000	-	3	2	-	83.30
8.	4500	-	3	3	-	100.00

In- vivo anti-inflammatory bioassay was carried out in carrageenan induced paw edema volume of *Swiss albino* mice by giving the oral administration of reference drug and various treatments (Figure 11). A perusal of figure exhibited statistically ($P<0.001$) significant decreasing trend in reduction in paw edema volume in all the treated groups. However, in the control group it increases initially then starts decreasing. The trend may be ascribed to the development of natural immunity with time.

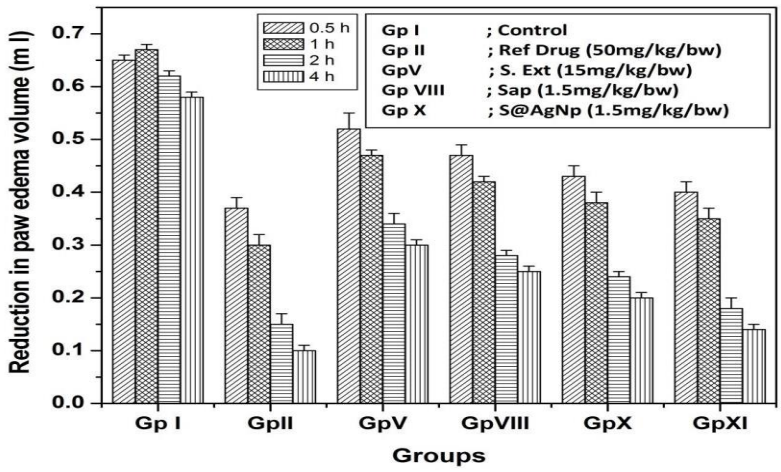


Figure 11. Decreasing trend in reduction in paw edema volume in various treated groups (mean ± standard deviation, n = 6)

Percentage inhibition of inflammation was calculated from a reduction in paw edema. values in each case and presented in Table 2.

Table 2. Percentage inhibition of inflammation in mice: Carrageenan induce hind paw edema

Treatment		Dose	Percentage inhibition Of Inflammation			
			0.5 h	1 h	2 h	4 h
Group II	Diclofenac sodium	50 mg/kg/bw	42.26	53.88	76.19	76.42
Group III	Seed extract	5 mg/kg/bw	10.70	19.42	35.82	35.98
Group IV	Seed extract	10 mg/kg/bw	13.84	22.05	40.29	40.88
Group V	Seed extract	15 mg/kg/bw	20.00	30.88	46.53	46.84
Group VI	Seed extract	20 mg/kg/bw	21.50	31.82	47.56	47.98
Group VII	Saponin	1.0 mg/kg/bw	24.23	35.23	50.23	52.23
Group VIII	Saponin	1.5 mg/kg/bw	27.69	38.23	55.22	56.10
Group IX	Saponin	3.0 mg/kg/bw	27.99	38.44	55.68	56.48
Group X	S@AgNps	1.5 mg/kg/bw	38.74	48.28	70.76	70.99

A time-dependent (0.5-4 h) increase in percentage inhibition of inflammation was noticed in all the treated groups. Antiinflammatory bio-efficacy (46.84%) at optimized dose (15 mg/kg/bw) of native seed extract was increased to 56.10% by bio-active principle (saponin) at considerable low dose (1.5 mg/kg/bw). The fact can be ascribed to the role of bio-active principle in the enhancement of the target bio-efficacy. Further, increase in the percentage inhibition (70.99%) was induced by bio-active principle loaded (saponin) silver nanoparticles at the same dose (1.5 mg/kg/bw). Thus overall enhancement in the anti-inflammatory bio-efficacy compared to the saponin treatment was found (26.54%) which can be ascribed to the combination of the contribution of the use of bio-active principle and green nanotech enhancement.

A well-defined mechanism of anti inflammation of metal nanoparticles has not been reported. In general, inflammation has been described as a biphasic process induced by pathogens and tissue injury. Initially, inflammatory signals are recognized by *toll-like receptors* which in turn activate *myeloid*

differentiation primary gene 88 [32]. These genes cause phosphorylation of IkB protein resulting into the release and translocation of NF-kβ protein into the nucleus. Transcription is upregulated causing the binding of NF-kβ protein to the inflammatory gene. This binding stimulates the various inflammatory promoting factors (cytokines, Interleukin, IFN–γ: interferon-gamma, TNF α: tumor necrosis factor-α, and cycloxygenase-2 enzymes) [33]. Therefore, control of release proinflammatory factors finally inhibits inflammation via down regulation of COX-2 enzyme and NF-kβ protein. An attempt has been made to explain the observed incremental enhancement in antiinflammatory bio-efficacy induced by bioactive principle (saponin) and saponin loaded silver nanoparticles. The present study reports the anti-inflammatory activity of saponin and S@AgNp$_s$ in terms of percentage inhibition 56.10% and 70.99% respectively. Histological investigations of the saponin and S@AgNp$_s$ treated groups provide support to the incremental improvement in the anti-inflammatory bioefficacy. Group I (control) showed damaged serosa, muscularis layer, villi tips and crypts (Figure 12 A).

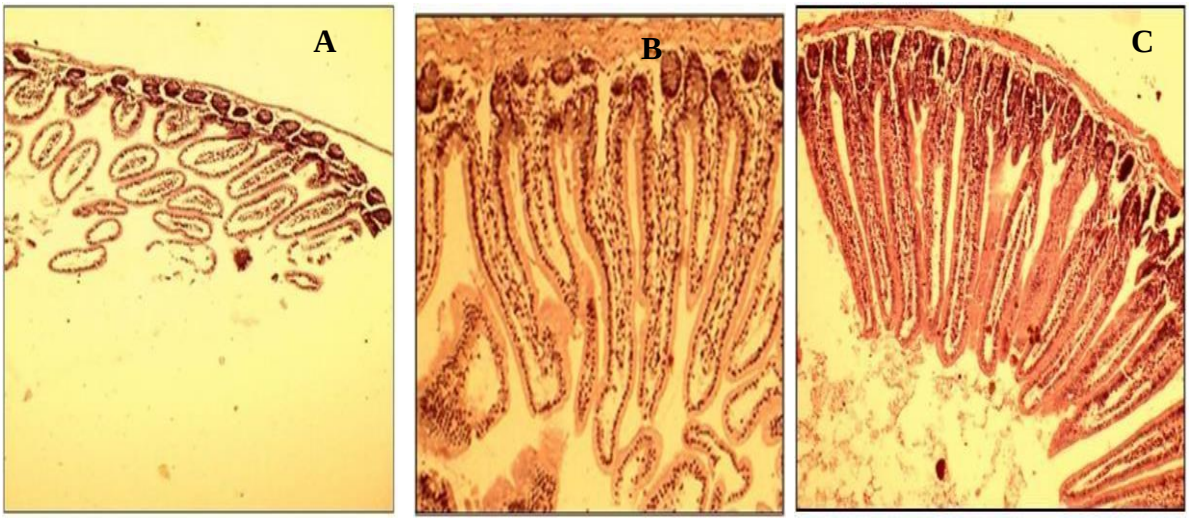


Figure 12. T.S. of intestine: Control group (A) Saponin treated group (B), S@AgNps treated group (C) at an optimum dose of 1.5 mg/kg/bw

Figure 12 B of group VIII (saponin) muscularis, less inflammation and describes comparatively well intact serosa, fragmented villi. Figure 12 C of group IX respectively. Histological investigations of the saponin and (S@AgNp$_s$) shows significantly reduced paw edema with more uniform serosa and muscularis layer with large numbers of villi. The fact highlights S@AgNp$_s$ are better, safe and non-toxic candidate compared to the treatment (saponin) for the antiinflammatory property.

Conclusion

The present work provides confirmatory scientific evidence for anti-inflammatory bio-efficacy in the seeds of the plant *M. longifolia*. The observed bio-efficacy has been assigned to the family of four saponins, explored in the seed extract of the target plant. The synergistic reduction potential of the saponin of the plant has been found strong enough for the synthesis of S@AgNp$_s$ in a simple and efficient green method. Promising statistically significant

enhancement in the anti-inflammatory bioefficacy has been successfully induced S@AgNp$_s$. The role of the combination of bio-active principle (saponin) and nano sizing in the enhancement in the target bioefficacy has been assigned to the use of bioactive principle, high surface area to volume ratio, reasonable stability, bio-compatibility and astonishing optical properties related to surface plasmon resonance. Overall, phenomenon allows accumulation and penetration of nano drug into living tissues comparatively deeper. Anti-inflammatory properties of silver also add to the enhancement in the inflammatory property. Thus, S@AgNp$_s$ prepared from the seed extract of the plant *M. longifolia* seem to be an ideal candidate for the development of alternative and complimentary herbal nanomedicine for anti-inflammation.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Acknowledgments

This work was supported by the University Grants Commission (UGC), New Delhi (India) vide fellowship No.ET/UGC Res. Fellowship/2136. Authors are thankful to Prof. P.K. Kalra, Director, and Prof. Sahab Dass, Head, Department of Chemistry, Dayalbagh Educational Institute Agra for extending all the necessary facilities and motivation to carry out the research.

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How to cite this manuscript: Mukti Sharma, Saurabh Yadav, Man Mohan Srivastava, Narayanan Ganesh, Shalini Srivastava* . Promising anti-inflammatory bio-efficacy of saponin loaded silver nanoparticles prepared from the plant Madhuca longifolia. *Journal of Medicinal and Nanomaterials Chemistry*, 4(4) 2022, 313-326. DOI: 10.48309/JMNC.2022.4.5